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The *Arabidopsis* cyclin-dependent kinase-activating kinase CDKF;1 is a major regulator of cell proliferation and cell expansion but is dispensable for CDK activation

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Summary

Cyclin-dependent kinases (CDKs) play an essential role in cell cycle regulation during embryonic and post-embryonic development of various organisms. Full activation of CDKs requires not only binding to cyclins but also phosphorylation of the T-loop domain. This phosphorylation is catalysed by CDK-activating kinases (CAKs). Plants have 2 distinct types of CAKs, namely, CDKD and CDKF; in *Arabidopsis*, CDKF;1 exhibits the highest CDK kinase activity *in vitro*. We have previously shown that CDKF;1 also functions in the activation of CDKD;2 and CDKD;3 by T-loop phosphorylation. Here, we isolated the knockout mutants of *CDKF;1* and showed that they had severe defects in cell division, cell elongation and endoreduplication. No defect was observed during embryogenesis, suggesting that CDKF;1 function is primarily required for post-embryonic development. In the *cdkf;1* mutants, T-loop phosphorylation of CDKA;1, an orthologue of yeast Cdc2/Cdc28p, was comparable to that in wild-type plants, and its kinase activity did not decrease. In contrast, the protein level and kinase activity of CDKD;2 were significantly reduced in the mutants. Substitution of threonine 168 with a nonphosphorylatable alanine residue made CDKD;2 unstable in *Arabidopsis* tissues. These results indicate that CDKF;1 is dispensable for CDKA;1 activation but is essential to maintain a steady-state level of CDKD;2, thereby suggesting a quantitative regulation of a vertebrate-type CAK in a plant-specific manner.

Introduction

In eukaryotes, cell cycle progression is regulated by cyclin-dependent serine/threonine protein kinases (CDKs). The kinase activity of CDKs is controlled by binding to cyclins, and it is maximised when the conserved threonine residue within the T-loop is phosphorylated by CDK-activating kinases (CAKs). The mammalian CAK, which is termed CDK7, is activated by binding to cyclin H (Fisher and Morgan, 1994; Labbé *et al*., 1994; Mäkelä *et al*., 1994). The RING finger protein MAT1 also interacts with CDK7 and functions in the stabilization of the CDK7-cyclin H complex (Devault *et al*., 1995; Tassan *et al*., 1995). CDK7 phosphorylates not only CDKs but also the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II. In fact, CDK7 has been identified in the general transcription factor TFIIH involved in the initiation and elongation of transcription (Serizawa *et al*., 1995; Shiekhattar *et al*., 1995). The *cdk7* null mutants of *Drosophila* were lethal before or soon after the initiation of pupation (Larochelle *et al*., 1998). Furthermore, in *Caenorhabditis elegans*, the RNA-mediated interference of *CDK7* resulted in an embryonic lethal phenotype (Wallenfang and Seydoux, 2002). Rossi *et al*. (2001) showed that the disruption of MAT1 led to peri-implantation lethality in mice. These reports indicate that CDK7 activity is indispensable during early developmental stages in animals.

In fission yeast, 2 kinases are involved in Cdc2 phosphorylation and activation (Buck *et al*., 1995; Damagnez *et al*., 1995; Hermand *et al*., 1998; Hermand *et al*., 2001; Saiz and Fisher, 2002). Mcs6 is an orthologue of CDK7 and has both CDK and CTD kinase activities. The lethality of the *mcs6* null mutants indicates that it plays an

essential role in cell division and/or basal transcription (Buck *et al*., 1995). Another CAK, named Csk1, phosphorylates Cdc2 but not CTD (Hermand *et al*., 1998). Interestingly, it also phosphorylates and activates Mcs6 as a CAK-activating kinase (CAKAK) (Hermand *et al*., 1998; Hermand *et al*., 2001), suggesting the existence of a CDK phosphorylation cascade (Csk1-Mcs6-Cdc2) in fission yeast. Cak1p of budding yeast is distantly related to CDK7 or Mcs6 and exhibits CAK activity but not CTD kinase activity (Espinoza *et al*., 1996; Kaldis *et al*., 1996). The CDK7 orthologue Kin28p is known to function as a CTD kinase, but it has no CAK activity (Cismowski *et al*., 1995; Valay *et al*., 1995). Therefore, in budding yeast, 2 distinct kinases are associated each with CDK and CTD phosphorylation.

Plant CDKs are classified into 6 types, namely, CDKA–CDKF (Vandepoele *et al*., 2002). CDKA is assumed to be an orthologue of yeast Cdc2/Cdc28p and regulates the G1-to-S and G2-to-M phase progression (Magyar *et al*., 1997). In *Arabidopsis*, *CDKA;1* is the sole gene that encodes CDKA and is expressed throughout the cell cycle (Menges *et al*., 2005; Vandepoele *et al*., 2002). The *cdka;1* null mutants were defective in pollen development, showing a gametophyte lethal phenotype (Iwakawa *et al*., 2006; Nowack *et al*., 2006). Hemerly *et al*. (2000) showed that *Arabidopsis* plants overexpressing a dominant negative form of CDKA;1 had severe defects during embryogenesis. These reports indicate that CDKA;1 has an essential function in early developmental stages such as gametogenesis and embryogenesis. It has also been reported that CDKA;1 plays an important role in post-embryonic development. Tobacco seedlings overexpressing a dominant negative form of *Arabidopsis* CDKA;1 consisted of larger and fewer cells

(Hemerly *et al*., 1995), and *Arabidopsis* mutants with the weak *cdka;1* allele showed a dwarf phenotype with fewer and larger leaf cells (Dissmeyer *et al*., 2007). However, in both cases, plants underwent normal morphogenesis with normal developmental timing. CDKBs are plant-specific CDKs, and their expression is restricted from the late S to the M phase (Porceddu *et al*., 2001). CDKB is further divided into 2 subgroups: CDKB1 and CDKB2. In *Arabidopsis*, there are 2 genes for each type of CDKB: *CDKB1;1* and *CDKB1;2* and *CDKB2;1* and *CDKB2;2* (Vandepoele *et al*., 2002). Transgenic plants overexpressing a dominant negative mutant of *CDKB1;1* produced less number of stomata with abnormal shape (Boudolf *et al*., 2004); they also showed a higher level of DNA ploidy, demonstrating that CDKB1 is required to suppress endoreduplication (Boudolf *et al*., 2004). On the other hand, the up- or downregulation of *CDKB2* caused severe defects in meristem functions. In overexpression or knock-down lines, the shoot apical meristem (SAM) contained considerably fewer and larger cells, and the strict organisation into 3 distinct cell layers was disrupted (Andersen *et al*., 2008). This indicates the differential role of CDKB2 in meristem organisation. C-type and E-type CDKs are assumed to regulate basal transcription, rather than cell division, by phosphorylating the CTD of RNA polymerase II (Cui *et al*., 2007; Fülöp *et al*., 2005; Joubès *et al*., 2001; Wang and Chen, 2004). Loss of CDKC;1 and CDKC;2 function resulted in altered leaf and flower growth (Cui *et al*., 2007). The *Arabidopsis cdke;1* mutants had defects in the specification of stamen and carpel identities and in the proper termination of stem cells in the floral meristem (Wang and Chen, 2004). Therefore, although CDKC and CDKE do not act upon CDKs, they are involved in plant growth

and development.

CDKD and CDKF were found to exhibit CAK activity in rice, *Arabidopsis* and *Euphorbia* (Chao *et al*., 2007; Umeda *et al*., 1998; Yamaguchi *et al*., 1998). CDKD is an orthologue of vertebrate-type CAKs, and *Arabidopsis* has 3 *CDKD* genes, namely, *CDKD;1–CDKD;3*. The CDK kinase activity of CDKD;3 was higher than that of CDKD;2, whereas CDKD;2 had higher CTD kinase activity than CDKD;3 (Shimotohno *et al*., 2003). CDKD;2 forms a stable complex with the H-type cyclin CYCH;1 (Shimotohno *et al*., 2004; 2006). CDKD;1 showed neither CDK nor CTD kinase activity *in vitro* (Shimotohno *et al*., 2004). *Arabidopsis* has a single gene for CDKF, named *CDKF;1*, which has been identified as a suppressor of the *cak1* mutation in budding yeast (Umeda *et al*., 1998). It shows similarity to CDK7 only in restricted domains, and unlike vertebrate-type CAKs, it has CDK-activating kinase activity but no CTD kinase activity *in vitro* (Umeda *et al*., 1998). CDKF;1 exhibited significant activity towards CDKA;1 in *Arabidopsis* root protoplasts, and this activity was dependent on T-loop phosphorylation of CDKF;1 (Shimotohno *et al*., 2006). A striking feature was that CDKF;1 also phosphorylates CDKD;2 and CDKD;3 and functions as a CAKAK, thereby regulating basal transcription and CDK activation (Shimotohno *et al*., 2004; Umeda *et al*., 2005). However, despite these *in vitro* analyses and transient assays with root protoplasts, the physiological function of CDKF;1 remains unknown.

Here, we conducted phenotypic analyses with knockout mutants of *CDKF;1*. They showed defects in cell division and cell expansion, leading to severe growth retardation in both shoots and roots. However, unlike CAKs in yeast and animals, the *cdkf;1*

mutants did not show any defect in early developmental processes, including embryogenesis. In the mutants, the protein level and kinase activity of CDKD;2 were significantly reduced, while those of CDKA;1 did not change compared to that of wild-type plants. We propose that CDKF;1 plays an important role in post-embryonic development by regulating the protein stability of CDKD;2.

Results

Expression pattern of CDKF;1

To understand the functional role of CDKF;1 in plant development, we first examined the expression pattern by using the β-glucuronidase (GUS) gene fused to the 2-kb promoter region of *CDKF;1*. However, no signal was detected in the transgenic plants, probably due to the lack of essential regulatory element(s) that promote(s) gene expression. Therefore, we generated another reporter line that expressed the CDKF;1-GUS fusion protein under the control of the *CDKF;1* promoter. As shown in Figures 1a and 1b, almost all tissues of seedlings were stained by incubation in a GUS-staining buffer for 4 h. This uniform expression pattern indicates that CDKF;1 may function not only in cell proliferation but also in cell expansion. To determine the tissues where *CDKF;1* is highly expressed, we then incubated the samples for a shorter period. Our results showed that actively dividing tissues were mainly stained, such as the apical meristems of shoots and roots, lateral root primordia, emerging lateral roots and vascular bundles (Figures 1c, 1d and 1i-k). The GUS expression was also observed in young flowers, especially in the stigma, pistil basal regions and vascular bundles of

petals (Figures 1e and 1f). In anthers, the expression was restricted to pollen grains (Figure 1g). GUS expression in siliques was observed only in the basal region (Figure 1h).

To analyse the temporal expression pattern of CDKF;1 during the cell cycle, an *Arabidopsis* MM2d cell culture was synchronised with aphidicolin. Flow cytometry analysis revealed a prominent peak of cells in G2, 4 h after aphidicolin removal (Figure 1l). This indicated that the majority of cells proceeded synchronously through the S phase. Reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that *CDKB2;1* transcripts, which accumulate from the G2 to the M phase (Kono *et al*., 2003; Mészáros *et al*., 2000), started to increase after 8 h (Figure 1l), indicating that cells at this stage were in the G2 phase. After 16 h, *CDKB2;1* expression began to decrease, and the G1 phase cells started to accumulate. We observed an elevated level of *CDKF;1* transcripts from 8 to 16 h (Figure 1l), suggesting that *CDKF;1* expression is upregulated from the G2 to the M phase. However, immunoblotting analysis showed that the CDKF;1 protein was constantly accumulated throughout the cell cycle (Figure 1l), indicating that the CDKF;1 function is not restricted to a specific phase of the cell cycle.

Isolation of the **cdkf;1** *mutants*

To reveal the *in vivo* function of CDKF;1, we identified *Arabidopsis* mutants from T-DNA insertion collections and named them *cdkf;1-1* and *cdkf;1-2*. These mutants have T-DNA insertions in the second exon of *CDKF;1* (Figure 2a). Both mutants exhibited the same phenotype, and introduction of the genomic fragment containing the

CDKF;1 gene restored the phenotype of *cdkf;1-1* mutants (Supplemental Figure 1a).

Next, we report the representative results obtained for the *cdkf;1-1* mutants. Total RNA was isolated from the *cdkf;1-1* mutants that were homozygous for T-DNA insertions and subjected to RT-PCR (Figure 2b). We could amplify the cDNA upstream of the T-DNA insertion site but not downstream of or encompassing the T-DNA insertion site. Immunoblotting analysis with the anti-CDKF;1 antibody that recognises the carboxy-terminal peptide did not detect intact CDKF;1 in the *cdkf;1-1* mutants (Figure 2b). This indicates that the mutants might produce transcripts with a truncation at the 3′-terminus. Next, we conducted rapid amplification of cDNA ends (3′-RACE) and isolated the full-length cDNA produced in the *cdkf;1-1* mutants. The cDNA, designated *mCDKF;1*, consisted of a partial *CDKF;1* lacking the C-terminal portion, 5 bp of an unknown sequence and 47 bp of the T-DNA end including the left border (Supplemental Figure 1b). The open reading frame (ORF) that ended at the stop codon within the T-DNA encodes N-terminal 379 amino acids of CDKF;1, followed by 16 amino acids of unrelated peptide (Supplemental Figure 1c). To examine the functionality of *mCDKF;1*, we expressed it in budding yeast GF2351 cells that carry a temperature-sensitive mutation in the *Cak1* gene. As shown in Figure 2c, the full-length *CDKF;1* could rescue temperature sensitivity, as reported previously (Umeda *et al*., 1998), but the cells expressing *mCDKF;1* did not grow at 37°C. This suggests that the *cdkf;1-1* mutants do not produce an active enzyme and thus are knockout mutants.

The* cdkf;1-1 *mutants are defective in cell division, cell elongation and

endoreduplication

Homozygous *cdkf;1-1* mutants did not generate inflorescence under growth conditions used in our study. Hence, we analysed the homozygous mutants segregated from *CDKF;1/–* plant seeds. They grew slowly and produced smaller, wavy leaves with abnormal serration (Figures 3a and 3b). Microscopic observations showed that both the epidermal and mesophyll cells were smaller than those of wild-type plants (Figure 3c). The number of epidermal cells in the first leaves was reduced to 36%, and the cell size was reduced to approximately one-half; as a result, the leaf blade area was reduced to 18% of that of the wild-type plants (Table 1). Sections of shoot apices indicated that the shoot apical meristem occupied only a limited area in the mutants (Figure 3d). These results suggest that *cdkf;1-1* mutants are defective in both cell division and cell elongation.

We found that under dark conditions, hypocotyl elongation was severely inhibited in the *cdkf;1-1* mutants (Supplemental Figures 2a and 2b). Epidermal cells in the mutant hypocotyl were not becoming longer in the dark, indicating an inhibition of cell elongation (Supplemental Figure 2c). GUS expression in the *pCDKF;1-CDKF;1::GUS* seedlings was not observed in the hypocotyl under light conditions, but it was detected in the upper portion of hypocotyls grown in the dark (Supplemental Figure 2d). This suggests that CDKF;1 is required for hypocotyl cell elongation in response to dark conditions.

To examine whether endoreduplication is also affected in the *cdkf;1-1* mutants, we analysed the DNA ploidy level in mature first leaves. As shown in Figure 3e, the ratio of

2C and 4C cells increased approximately 2-fold in the mutants, while the 8C cell population was drastically reduced. This indicates that the *cdkf;1-1* leaves were defective in endoreduplication. This observation was supported by the finding that the average number of trichome branching in the mutants was 2 (Table 1), probably due to a lower level of endoreduplication during trichome development. Note that the CDKF;1-GUS fusion protein accumulated in young trichomes but disappeared in the mature stage (Figure 3f), suggesting that CDKF;1 may be involved in the trichome development via controlling endoreduplication.

Cell division but not cell differentiation is inhibited in the* cdkf;1-1 *roots

Root growth of the *cdkf;1-1* mutants was retarded, and it stopped around 4 days after germination (DAG) (Figures 4a and 4b). As a result, the mutants produced primary roots with lengths of 2–3 mm and fewer lateral roots, which started to elongate when the primary roots stopped growing (Figure 4a). The meristematic zone in the root tips was shorter at 4 DAG and was almost lost at 8 DAG (Supplemental Figure 3). To monitor cell proliferation, we introduced *pCYCB1;1–CYCB1;1::GUS*—a G2/M phase marker—into the *cdkf;1-1* mutants. As shown in Figure 4c, the patchy pattern of GUS signals almost disappeared in the root meristem of 4 DAG mutants, suggesting that CDKF;1 is prerequisite to maintain cell proliferation during the post-embryonic development of roots. We also observed a reduction in the number of columella cell layers in the *cdkf;1-1* mutants (Figure 4d; 4.29 ± 0.46 in wild type, n = 38; 3.12 ± 0.33 in *cdkf;1-1*, n = 50), indicating reduced cell division in columella initial cells.

Aberrant root growth is often accompanied by a failure to maintain cell differentiation. Therefore, we analysed the expression pattern of several cell type-specific markers in the *cdkf;1-1* mutants. *SCARECROW* (*SCR*) is a marker gene for the endodermis, the cortex/endodermal initial cells and the quiescent centre (QC) (Di Laurenzio *et al*., 1996). As shown in Figure 4e, *SCR* expression did not change in the *cdkf;1-1* mutants, indicating that there was no defect in the radial patterning of roots. The expression pattern of the QC marker QC46 was almost the same, but the area of GUS-stained cells was slightly larger in the mutants (Figure 4e). We then examined the identity of columella cells by visualising starch granules with lugol staining. As noted in wild-type plants, starch granules were observed in mature columella cells but not in the columella initials of the mutants (Figure 4e), suggesting that initial cells maintain their pluripotent state regardless of severe defects in cell division. These results indicate that CDKF;1 is not involved in cell differentiation, but it plays an essential role in cell division during root development.

No obvious defect was observed during embryogenesis of the* cdkf;1-1 *mutants

To examine whether embryogenesis is affected in the *cdkf;1-1* mutants, we observed mature embryos in seeds from the heterozygous mutants. Microscopically, we could not discern *cdkf;1*/– embryos from wild-type or *cdkf;1*/+ embryos. We then observed cotyledons of mature embryos in wild-type and mutant parental seeds, but again found no difference in cell number or cell area (Supplemental Figure 4a). Moreover, in embryonic roots in seeds from *cdkf;1-1*/+ plants, we found no defect in the number of

columella cell layers and the radial patterning of cell files (Supplemental Figure 4b). These results suggest that embryogenesis is not aberrant in the *cdkf;1-1* mutants.

CDKA and CDKB activities are maintained in the **cdkf;1-1** *mutants*

To verify which *Arabidopsis* CDK(s) are *in vivo* targets of CDKF;1, the protein level and kinase activity of CDKs were compared between wild-type plants and the *cdkf;1-1* mutants. First, we examined A- and B-type CDKs, which directly regulate cell cycle progression (Figure 5a). Unexpectedly, the activity of CDKA;1 was not reduced in the *cdkf;1-1* mutants. Immunoblotting with anti-CDKB antibodies showed that the protein levels of CDKB1 and CDKB2 were higher in the mutants, but there was no difference in their kinase activities. This indicates that the CDKB kinase activity per molecule might be lower in the mutants, but the total activity was comparable to that in wild-type plants. As described above, because wild-type and mutant seedlings showed differences in development, we could not rule out the possibility that these results might reflect a difference in the state of cell differentiation. Therefore, we also examined the protein levels and activities of above-mentioned CDKs in calli, which are undifferentiated masses of dividing cells that are more uniform than seedlings. The results were almost the same as seedlings, and the kinase activities of CDKA, CDKB1 and CDKB2 in the mutants were comparable to those in wild-type calli (Figure 5b).

Next, to examine further the T-loop phosphorylation of CDKs, we monitored the phosphorylation state of CDKA;1 by using anti-phospho-Cdc2 (Thr161) antibody. This antibody recognised a protein with the same molecular weight as that of CDKA;1, while

the band disappeared in a protein extract preincubated with lambda protein phosphatase or depleted with anti-CDKA;1 antibody (Figure 5c). This result indicates that the antibody specifically recognised CDKA;1 phosphorylated within the T-loop. Immunoblotting analysis with this antibody revealed that the phosphorylation level of CDKA;1 in the *cdkf;1-1* mutants was comparable to that in wild-type plants (Figure 5d), suggesting that CDKA;1 may be phosphorylated and activated by CAK(s) other than CDKF;1 in the mutants. Anti-phospho-Cdc2 (Thr161) antibody did not recognise CDKB1 or CDKB2, thus we could not examine the phosphorylation state of CDKBs.

CDKF;1 controls the steady-state level of CDKD;2 by T-loop phosphorylation

We then investigated CDKD;2 and CDKD;3, which are also *in vitro* targets of CDKF;1 (Shimotohno *et al*., 2004; Umeda *et al*., 1998). In wild-type seedlings, we could detect CDKD;2 but not CDKD;3 in a protein extract (Figure 6a). Interestingly, the protein level of CDKD;2 significantly decreased in the *cdkf;1-1* mutants, and we detected only a trace level of kinase activity on the CTD substrate (Figure 6a). In calli, we again observed that the protein level of CDKD;2 but not that of CDKD;3 was reduced in the *cdkf;1-1* mutants (Figure 6b). Quantitative RT-PCR showed that the transcripts of both *CDKD;2* and *CDKD;3* were increased in the mutant seedlings, compared to those of wild-type (Figure 6c); this may indicate a compensation for the loss of CDKF;1. These results suggest that the translation efficiency or protein stability of CDKD;2 was reduced in the absence of CDKF;1.

To observe the accumulation of CDKD proteins in tissues, we generated

transgenic plants expressing the CDKD::GUS fusion protein under the control of the *CDKD* promoter. In wild-type roots, the CDKD;2::GUS fusion protein was accumulated in the meristematic and elongation zones, but it disappeared in the root tips of the *cdkf;1-1* mutants (Figure 7a). In contrast, the CDKD;3::GUS fusion protein remained in the root tips of the mutants, although the expression domain was restricted to the distal region because of the reduced meristem size, as described above (Figure 7a). Similar results were also obtained in lateral root primordia, young leaves and hypocotyls (Figure 7a). Note that both *CDKD;2::GUS* and *CDKD;3::GUS* mRNA levels slightly increased in the *cdkf;1-1* mutants compared to those in wild-type plants (Figure 7b). Therefore, our results indicate that CDKF;1 controls the steady-state level of CDKD;2 but not CDKD;3 in *Arabidopsis* tissues.

To examine whether the control of the CDKD;2 protein level was dependent on phosphorylation by CDKF;1, we substituted the conserved threonine residue (T168) with alanine within the T-loop in the *pCDKD;2-CDKD;2::GUS* construct and introduced it into the wild-type plants. As shown in Figure 8, GUS expression was considerably weaker in the T168A variant than in the wild-type plants in both the shoots and roots. Five transgenic lines showed similar results, and the *CDKD;2::GUS* transcripts showed the same level of accumulation in the wild-type plants and the T168A mutant (Supplemental Figure 5). These results suggest that T-loop phosphorylation by CDKF;1 is required to maintain the steady-state level of the CDKD;2 protein.

Discussion

We showed that the *cdkf;1-1* mutants exhibited defects in cell division in both the shoots and roots. We also found that they were defective in endoreduplication and cell elongation in leaves and dark-grown hypocotyls. These results indicate that the CDKF;1 function is not limited to cell cycle control, but it plays multiple roles in the endocycle and in the process of cell growth. The CDKF;1-GUS fusion protein under the control of the *CDKF;1* promoter was expressed in various tissues with a higher accumulation in the meristems of shoots and roots, dark-grown hypocotyls, young flowers and pollen grains. Although we do not know the functionality of the fusion protein, such a diverse expression pattern may support the multiple role of CDKF;1 during plant development.

The *cdkf;1-1* mutants appeared to complete embryogenesis successfully, suggesting that CDKF;1 is primarily required for post-embryonic development. In seedlings, the kinase activity of CDKA;1 was almost the same as that in wild-type plants. This indicates that the characteristic phenotype of the *cdkf;1-1* mutants originated from disordered functions of factor(s) other than CDKA;1. In line with this assertion, it was noted that *cdka;1* null mutants failed to progress through the second mitotic division during male gametophyte development (Iwakawa *et al*., 2006; Nowack *et al*., 2006), while there was no defect in *cdkf;1-1* mutant pollen. Moreover, transgenic plants overexpressing a dominant-negative type of *CDKA;1* produced fewer but larger leaf cells (Hemerly *et al*., 1995), while both the cell number and cell size were reduced in the *cdkf;1-1* mutants. We found that CDKB1 and CDKB2 kinase activities per molecule were lower in the mutants, but the total CDKB activity was comparable to that

in wild-type plants. Therefore, it is unlikely that a dysfunction of CDKB caused the *cdkf;1-1* mutant phenotype. We assume that CDKF;1 may have other unknown target(s) that function in regulating cell division, cell elongation and endoreduplication during post-embryonic development.

CDKF;1 is able to suppress CAK mutation in budding and fission yeasts, and it phosphorylates and activates human CDK2 *in vitro* (Shimotohno *et al*., 2004; Umeda *et al*., 1998). Moreover, we previously showed that *CDKF;1* overexpression in *Arabidopsis* root protoplasts activates CDKA;1, suggesting that CDKF;1 is involved in activating endogenous CDKs *in vivo* (Shimotohno *et al*., 2006). However, in the present study, we found that CDKA;1 was not downregulated in the *cdkf;1-1* mutants. It is unlikely that CDKF;1 activates CDKA;1 only in specific tissues because CDKA;1 and CDKF;1 are expressed in largely overlapping tissues (Hemerly *et al*., 1993; this study). Mechanisms in which the lack of a particular cell cycle regulator is compensated by other proteins have been reported in animals. For instance, Aleem *et al*. (2005) showed that in $CDK2^{-/-}$ mice, CDK1 compensated for the loss of CDK2 function by forming an active CDK1-cyclin E complex. During mouse embryonic development, 3 D-type cyclins are expressed in mutually exclusive cell types. However, mice lacking 2 of the 3 D-type cyclins could develop normally until late gestation because the remaining cyclin was ubiquitously expressed in the whole embryo, thereby losing its tissue specificity and compensating for the loss of the other 2 D-type cyclins (Ciemerych *et al*., 2002). In the *cdkf;1-1* mutants, it is also likely that the redundant function of CDKDs might be sufficient for the activation of CDKA;1. Among the 3 CDKD homologues, *CDKD;3*

exhibited a higher suppression activity in the *S. cerevisiae* mutant GF2351 (Shimotohno *et al*., 2003). Although the transcript and protein levels of CDKD;3 were reported to be low in plant tissues (Shimotohno *et al*., 2003; this study), its activity might be sufficient to fully phosphorylate and activate CDKA;1, without the CDKF;1 function.

The protein level and kinase activity of CDKD;2 were significantly decreased in the *cdkf;1-1* mutants. In addition, the expression of a nonphosphorylatable form (T168A) of CDKD;2 was reduced compared to that of the wild-type protein, regardless of the sustained level of transcripts. These results indicate the important role of T-loop phosphorylation in maintaining the steady-state level of CDKD;2. However, the proteasome inhibitor MG132 did not stabilise CDKD;2::GUS in the *cdkf;1* mutants, and CDKD;2 (T168A) fused to GFP was localised in the cytoplasm and nuclei in the same manner as the wild-type protein (data not shown; Shimotohno *et al*., 2004). Therefore, we assume that the T-loop phosphorylation by CDKF;1 controls the protein stability of CDKD;2 rather than protein degradation via the ubiquitin-proteasome pathway or a change in subcellular localisation.

Quantitative regulation of CAK components has also been described in other organisms. In animals, MAT1 is known to stabilise the CAK complex consisting of CDK7 and cyclin H (Devault *et al*., 1995; Fisher *et al*., 1995; Tassan *et al*., 1995). It was found that the loss of MAT1 resulted in the reduction of protein levels of CDK7 and cyclin H, suggesting that MAT1 regulates the steady-state level of the CAK complex (Rossi *et al*., 2001). The cyclin H-CDK7 dimer is also activated by the T-loop phosphorylation (Devault *et al*., 1995; Fisher *et al*., 1995; Tassan *et al*., 1995), but it

remains unknown whether this phosphorylation is associated with the control of their protein levels. The amount of the budding yeast protein Kin28p, an orthologue of CDK7, was reduced in a strain of *cak1* mutants, in which Kin28p was not subjected to activation by phosphorylation (Espinoza *et al*., 1998). Further, in the absence of a MAT1 orthologue, Tfb3p, substitution of the conserved threonine residue within the T-loop with alanine led to a significant reduction in the Kin28p level (Kimmelman *et al*., 1999). These observations support our proposal that in *Arabidopsis*, the protein stability of CDKD;2 depends on the T-loop phosphorylation by CDKF;1.

We previously reported that CDKF;1 phosphorylates not only CDKD;2 but also CDKD;3 (Shimotohno *et al*., 2004); however, the amount of CDKD;3 in calli was almost identical between the wild-type plants and *cdkf;1-1* mutants. CDKD;2 and CDKD;3 phosphorylate both CDK and CTD, albeit with different preferences: the CDK kinase activity of CDKD;3 is higher than that of CDKD;2, whereas CDKD;2 has higher CTD kinase activity than CDKD;3 (Shimotohno *et al*., 2003). Therefore, our results indicate that CDKF;1 controls basal transcription by regulating the steady-state level of CDKD;2 rather than CDKD;3 and that some phenotypes observed in the *cdkf;1-1* mutants might be caused by transcription defects. For unknown reasons, we were unable to overexpress a constitutive-active form (T168D) of CDKD;2 in the *cdkf;1-1* mutants; thus, to test the contribution of CDKD;2 function in the *cdkf;1* mutants, we may need to use a different approach. In *Drosophila*, the expression of a dominant-negative form of CDK7 severely delayed the onset of zygotic transcription during early embryogenesis, but did not alter the timing of the first 13 embryonic

nuclear cycles, which occur without *de novo* transcription (Leclerc *et al*., 2000). This result indicated a major function of CDK7 in the transcriptional control in animal cells. Recently, *Arabidopsis* mutants of cyclin T have been shown to exhibit embryonic lethality (Cui *et al*., 2007). Plant cyclin T is a binding partner of CDKC, an orthologue of mammalian CDK9 that functions as a CTD kinase (Fülöp *et al*., 2005). Therefore, this report suggests an essential role of CTD kinases in early developmental processes. In budding yeast, Cak1p activates CDK9 orthologues, namely, Bur1 and Ctk1, via the phosphorylation of conserved threonine residues within the T-loop (Ostapenko *et al*., 2005; Yao *et al*., 2002), suggesting that *Arabidopsis* CDKF;1 may also control basal transcription through the phosphorylation of CDKC and CDKD;2. Studies using loss-of-function mutants of CDKC and CDKD and their associated cyclins are expected to reveal the indispensable function of CTD kinases during plant development.

In yeast and animals, the CDK activity is modified by the WEE1 kinase, which phosphorylates the ATP-binding site and inhibits activity (Featherstone *et al*., 1991; Parker *et al*., 1992). However, in *Arabidopsis*, the knockout mutants of *WEE1* showed no obvious phenotype when grown under normal growth conditions (De Schutter *et al*., 2007). This suggests that unlike in yeast and animals, WEE1 is not a critical CDK modifier in plants. In contrast, in the current study, we showed that CDKF has a crucial role in the post-embryonic development of *Arabidopsis*. A dissection of the regulatory mechanisms underlying the CAK activity and the identification of novel substrates of plant-specific CDKF will reveal how cell cycle regulation is integrated into developmental control in cooperation with various signalling pathways and

transcriptional networks.

Experimental procedures

Plant material

Arabidopsis thaliana (ecotype Col-0) was first grown at 23°C on an MSAR agar plate (Koncz *et al*., 1990) under short-day conditions (dark, 16 h; light, 8 h); it was then transferred onto soil and grown under continuous light conditions. Calli were generated from dark-grown hypocotyls (Kono *et al*., 2006). A suspension culture of the *Arabidopsis* cell line MM2d was maintained and synchronised using aphidicolin, as described by Menges and Murray (2002). A flow cytometry analysis was conducted with a Ploidy Analyzer (PARTEC, Münster, Germany).

Identification of T-DNA insertion mutants of CDKF;1

The T-DNA insertion mutants of *CDKF;1* were isolated from the collections of GABI-Kat and the Salk Institute; the seed stock numbers of *cdkf;1-1* and *cdkf;1-2* are GABI-Kat 315A10 and SALK_148336, respectively. The insertions were examined by genomic PCR with Ex Taq DNA polymerase (TaKaRa, Tokyo, Japan) by using a set of primers that hybridise to the T-DNA and *CDKF;1*: 5′-AACAACCGGCGACCAGTTGGAGTAT-3′ and 5′-CCCATTTGGACGTGAATGTAGACAC-3′ were used for *cdkf;1-1*, and 5′-AACAACCGGCGACCAGTTGGAGTAT-3′ and 5′-GGATTTTCGCCTGCTGGGGCAAACCAGCGT-3′ were used for *cdkf;1-2*. Each

line was backcrossed 3 times with wild-type plants. The 3′ end of the *CDKF;1* transcripts produced in the mutants was identified with a 3′ RACE System (Invitrogen, San Diego, CA, USA). The primers used for PCR amplification were as follows: the adapter primer and the abridged universal amplification primer, 5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′ and 5′-GGCCACGCGTCGACTAGTAC-3′, respectively, and the *CDKF;1*-specific primer, 5′-ACGAATACTTCTGGCGTG-3′. For the subsequent nested-PCR, the following 3 primers for the ORF of *CDKF;1* were used: 5′-GGATACTCATGGAGC-3′, 5′-GCTTCAGATGAAAACCAACAAGCTTAC-3′ and 5′-GATGGAGAAACCAGCGAACCACCAG-3′. The PCR products were purified, and their nucleotide sequences were determined.

RT-PCR

cDNA was synthesised with total RNA by using Superscript II reverse transcriptase (Invitrogen). In a total reaction volume of 20 µl, 1–2 µg of RNA was used. For PCR reactions, Ex Taq DNA polymerase (TaKaRa) and the primers listed in Supplemental Table 1 were used. The PCR conditions were as follows: 1 cycle at 94°C for 2 min; 25–35 cycles at 98°C for 10 s, at 55°C for 30 s and at 72°C for 1 min; and 1 cycle at 72°C for 7 min. Quantitative RT-PCR was conducted with the LightCycler system (Roche, Basel, Switzerland) by using SYBR Premix Ex Taq (TaKaRa). The PCR conditions were as follows: 1 cycle at 95°C for 5 min; 45 cycles at 95°C for 10 s, at 60°C for 10 s and at 72°C for 10 s.

Yeast complementation test

mCDKF;1 was PCR-amplified from the cDNA prepared from the *cdkf;1-1* mutants and cloned into the *Eco*RI site of pYX112. A complementation test was conducted with the budding yeast strain GF2351 (*MATa*, *civ1-4*, *ura3*, *leu2*, *trp1*, *lys2*, *ade2* and *ade3*) (Thuret *et al*., 1996). Transformants were incubated on a minimal medium lacking uracil at 27°C or 37°C for 4 days.

Plasmid construction for plant transformation

For a complementation test of the *cdkf;1* mutants, the genomic fragment of *CDKF;1* was amplified from 2032 bp upstream of the start codon to 1032 bp downstream of the stop codon; it was cloned into the GATEWAY entry vector pENTR/D-TOPO (Invitrogen). A recombination reaction was performed between the entry clone and the destination vector pGWB1 (Nakagawa *et al*., 2007) by using LR Clonase (Invitrogen). For expression analysis, the genomic fragments of *CDKF;1, CDKD;2* and *CDKD;3* were amplified from 2032, 2011 and 2000 bp upstream of the start codon, respectively, to the amino acids just before the stop codon. These fragments were cloned into pENTR/D-TOPO, and an LR reaction was performed with the destination vector pGWB3 (Nakagawa *et al*., 2007) to be in frame with the GUS gene. The T168A mutation in *CDKD;2* was introduced using a GeneTailor™ Site-Directed Mutagenesis System (Invitrogen).

Microscopy observations

A kinematic analysis of leaf growth was performed as described previously (De Veylder *et al*., 2001). Plants were grown on an MSAR agar plate for 15 days, and the seedlings were transferred to soil and grown for 10 days. Healthy first leaves were harvested and fixed in a solution of 90% ethanol and 10% acetic acid at 4°C overnight, hydrated through a graded series of ethanol and stored in water at 4°C. The samples were mounted on a glass slide and cleared with a chloral hydrate solution (71% chloral hydrate and 11% glycerol) during overnight incubation. Data were collected by scanning images of the abaxial epidermis located at 50% the distance between the tip and the base of the leaf blade, halfway between the midrib and the leaf margin. The images that included at least 40 cells in focus were edited using Photoshop Elements 2.0 (Adobe, San José, CA, USA). The epidermal cells in the edited image were counted, and the area of the edited image was measured using the image analysis program NIH Image 1.63. The average cell area was determined on the basis of these measurements. Next, the leaf blade area was measured, and the total number of epidermal cells on the abaxial side was estimated on the basis of the average cell area. The trichome branch number was counted on mature first leaves.

Roots were mounted on a glass slide in a chloral hydrate solution. Embryos obtained from dry seeds were soaked in water for at least 1 h and subsequently fixed and cleared as described previously (Malamy and Benfey, 1997). GUS staining was conducted as described by Umeda *et al*. (2000). For visualisation of starch granules, roots were incubated for 3 min in Lugol solution (Sigma, Brooklyn, NY, USA).

Immunoblotting and kinase assay

Immunoblotting was performed using an ECL Western Blotting Detection kit (GE Healthcare, Buckinghamshire, UK). Specific antibodies of *Arabidopsis* CDKs were used as described previously (Kono *et al.*, 2003; Shimotohno *et al*., 2003; Umeda *et al*., 1998). A polyclonal anti-CDKB1 antibody was raised against the carboxy-terminal FDSLDKSQF peptide of CDKB1;1. Commercial antibodies used include anti-actin antibody (MP Biomedicals, Morgan Irvine, CA, USA) and anti-phospho-Cdc2 (Thr161) antibody (Cell Signaling Technology, Danvers, MA, USA). For phosphatase treatment, 50 μg of protein extract was incubated with 200 U of lambda protein phosphatase (New England Biolabs, MA, USA) at 30°C for 1h. For kinase assay, 40 μg of protein extract was immunoprecipitated and subjected to kinase assay by using histone H1 (Roche) or GST-CTD as a substrate (Umeda *et al*., 1998).

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Supplemental Material

The following supplementary materials are available for this article online:

Supplemental Figure 1. Complementation of the *cdkf;1* mutants.

Supplemental Figure 2. Inhibition of hypocotyl elongation in the *cdkf;1-1* mutants.

Supplemental Figure 3. Root meristem size is reduced in the *cdkf;1-1* mutants.

Supplemental Figure 4. Embryogenesis is not defective in the *cdkf;1-1* mutants.

Supplemental Figure 5. RT-PCR analysis of *pCDKD;2-CDKD;2::GUS* transgenic plants.

Supplemental Table 1. Primers used for RT-PCR

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Figure legends

Figure 1. Spatio and temporal expression pattern of *CDKF;1*.

GUS staining of transgenic plants harbouring *pCDKF;1-CDKF;1::GUS*. (a, b) GUS staining of 12-day-old seedlings and 8-day-old roots, respectively, for 6 h. Bar = 5mm (a), 100 μm (b). (c) GUS staining of 12-day-old seedlings for 1 h. Bar = 1 mm. (d) Shoot apical meristem. Bar = 20 μm. (e) Flowers. Bar = 1 mm. (f) Magnified image of a flower. Bar = 1 mm. (g) Anther. Bar = 100 μm. (h) Young silique. Bar = 1 mm. (i) Primary root. Bar = 50 μm. (j) Lateral root primordium. Bar = 100 μm. (k) Emerging lateral root. Bar = 100 μm. (l) Flow cytometry analysis of *Arabidopsis* MM2d suspension culture (top), RT-PCR analysis of *CDKF;1*, *CDKB2;1* and β tubulin (*TUB4*) genes and immunoblotting of CDKF;1 (bottom). Samples were harvested at the indicated time after release from the aphidicolin block.

Figure 2. Isolation and characterisation of the *cdkf;1* mutants.

(a) A schematic diagram of the *CDKF;1* gene. Exons and introns are indicated by black boxes and solid bars, respectively. Open triangles represent the T-DNA insertion sites. Black arrowheads indicate primers used for RT-PCR. (b) Expression analysis of the *cdkf;1-1* mutant. RT-PCR was conducted with total RNA from whole seedlings (10 DAG) of wild-type plants or the mutants by using the indicated sets of primers. The amplified cDNAs were stained with ethidium bromide. Twenty micrograms of total protein was subjected to immunoblotting with anti-CDKF;1 antibody. (c) Complementation of the *S. cerevisiae* mutant GF2351. Transformants carrying each

plasmid were incubated at 27°C or 37°C for 4 days.

Figure 3. Shoot phenotypes of the *cdkf;1-1* mutants.

(a) 25-day-old and 45-day-old plants of wild-type and the *cdkf;1-1* mutants. Bars = 1 cm. (b) Magnified images of 25-day-old and 45-day-old plants of the *cdkf;1-1* mutants. Bar = 5 mm. (c) Leaf cells of 25-day-old plants. Bar = 100 μm. (d) Shoot apices of 7-day-old seedlings stained with toluidine blue. Bar = 5 μm. (e) Distribution of DNA ploidy in first leaves was measured with a Ploidy Analyser. (f) GUS staining of young and mature trichomes harbouring *pCDKF;1-CDKF;1::GUS*. Bar = 50 μm (top), 100 μm (bottom).

Figure 4. Inhibition of cell division in the *cdkf;1-1* roots.

(a) 8-day-old (top) and 27-day-old seedlings (bottom) of wild-type plants and *cdkf;1-1* mutants. PR, primary root; LR, lateral root. Bars = 1 cm (top) and 1 mm (bottom). (b) Primary root growth after germination. (c) Expression pattern of *pCYCB1;1–CYCB1;1::GUS* in root tips of wild-type plants and the *cdkf;1-1* mutants (4 DAG). Bar = 50 μm. (d) Root apices of 4-day-old seedlings. Yellow and red arrowheads indicate columella initials and mature columella cells, respectively. QC, quiescent centre. Bar = 25 μm. (e) Expression pattern of *pSCR-GUS* and *QC46-GUS* in wild-type plants and the *cdkf;1-1* mutants (4 DAG). Seedlings were GUS stained for 24 h (for *SCR*) or 18 h (for *QC46*). Starch granules in columella mature cells were visualised by lugol staining. Blue and yellow arrowheads indicate quiescent centre and

columella initials, respectively. Bars = 25 μm.

Figure 5. CDKA and CDKB activities are not reduced in the *cdkf;1-1* mutants.

(a, b) Protein levels and kinase activities of CDKA and CDKB in wild-type and the *cdkf;1-1* mutants. Protein extracts (10 μg for CDKA, 40 μg for CDKBs and 5 μg for actin) from 10-day-old seedlings (a) or calli (b) were subjected to immunoblotting with specific antibodies. A kinase assay was conducted with immunoprecipitates by using histone H1 as a substrate. (c) Detection of the phosphorylated form of CDKA;1. Protein extracts (60 μg) from calli were incubated with or without lambda protein phosphatase (left), or depleted with anti-CDKA;1 antibody (right). Then the samples were subjected to immunoblotting with anti-CDKA;1 or anti-phospho-Cdc2 (Thr161) antibody. CDKA-P indicates CDKA;1 phosphorylated within the T-loop. (d) Protein extracts (60 μg) from calli of wild-type plants and the *cdkf;1-1* mutants were subjected to immunoblotting with anti-phospho-Cdc2 (Thr161) antibody. Bands with an asterisk represent nonspecific cross-reactions with the antibody.

Figure 6. Protein level of CDKD;2 is reduced in the *cdkf;1-1* mutants.

(a) The protein levels and kinase activities of CDKD in wild-type plants and the *cdkf;1-1* mutants. Protein extracts (30 μg) from 10-day-old seedlings were subjected to immunoblotting with specific antibodies. A kinase assay was conducted with CDKD;2 immunoprecipitates by using GST-CTD as a substrate. (b) Protein levels of CDKD in calli of wild-type plants and the *cdkf;1-1* mutants. Protein extracts (30 μg) were

immunoblotted with specific antibodies. (c) Quantitative RT-PCR was conducted with total RNA from 10-day-old seedlings and the primer sets for *CDKD;2* and *CDKD;3*. The expression levels of *CDKD;2* and *CDKD;3* were normalized to *ACT8* (*ACTIN 8*). Expression levels in the *cdkf;1-1* mutants are indicated as relative values when those in wild-type plants were set for 1. Data are presented as mean ± SE of 4 samples.

Figure 7. Decrease in the steady-state level of CDKD;2 in the *cdkf;1-1* mutants.
(a) GUS staining of transgenic plants harbouring *pCDKD;2-CDKD;2::GUS* or *pCDKD;3-CDKD;3::GUS*. Shoots of seedlings (top row), hypocotyls (second row), primary root tips (third row) and lateral root primordia (bottom row). Bar = 1 mm (top row) and 100 μm (second to bottom row). (b) Quantitative RT-PCR was conducted with total RNA from 10-day-old seedlings and the primer sets for the *GUS* gene. The *GUS* expression levels were normalized to *ACT8*. Expression levels in the *cdkf;1-1* mutants are indicated as relative values when those in wild-type plants were set for 1. Data are presented as mean ± SE of 5 transgenic lines.

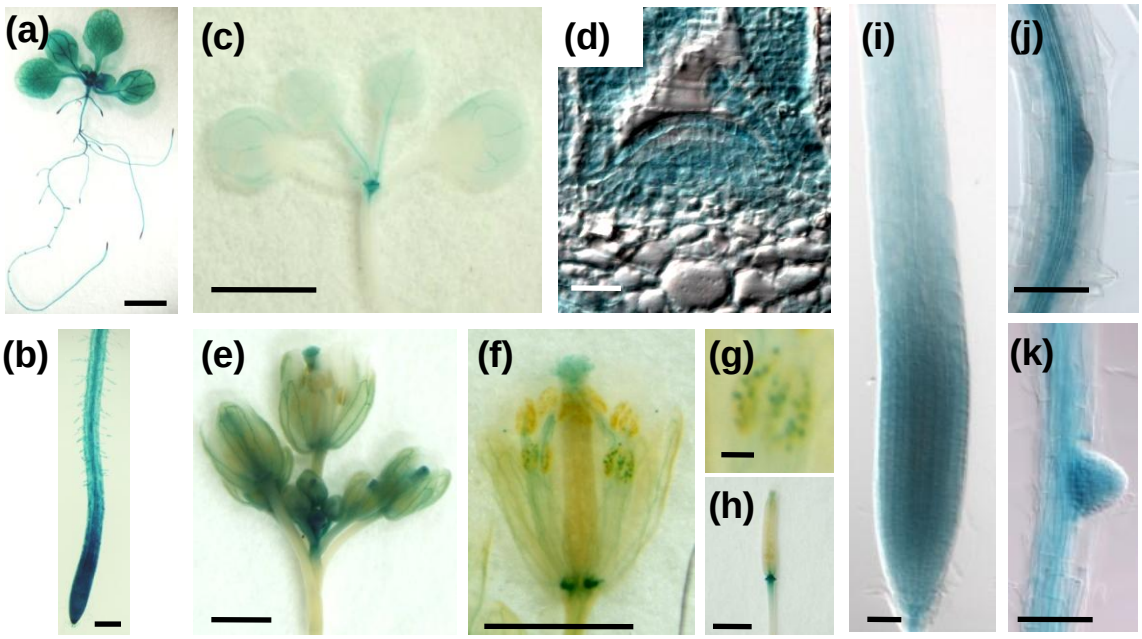
Figure 8. CDKD;2 with the T168A mutation is unstable in *Arabidopsis* tissues.
GUS staining of transgenic plants harbouring *pCDKD;2-CDKD;2::GUS* (WT) or *pCDKD;2-CDKD;2::GUS* (T168A) in the wild-type background. Shoots (left) and primary roots (right) of 10-day-old and 4-day-old seedlings, respectively. Bar = 1 mm (left) and 100 μm (right).

Table 1. Leaf blade area, cell area, cell number and trichome branch number in the first leaves of wild-type plants and *cdkf;1-1* mutants.

	Leaf blade area (mm^2)	Cell area (μm^2)	Cell number	Trichome branch number
WT	12.69 ± 3.61	4543 ± 908	2752 ± 332	2.89 ± 0.06
cdkf;1-1	2.36 ± 0.70	2362 ± 509	991 ± 147	1.96 ± 0.11

All measurements were performed using 25-day-old seedlings. Cell area and cell number were estimated for the abaxial epidermal cells. Data are presented as mean ± SE (n = 12).

Figure 1


(a)
(c)
(d)
(i)
(j)
(b)
(e)
(f)
(g)
(h)
(k)

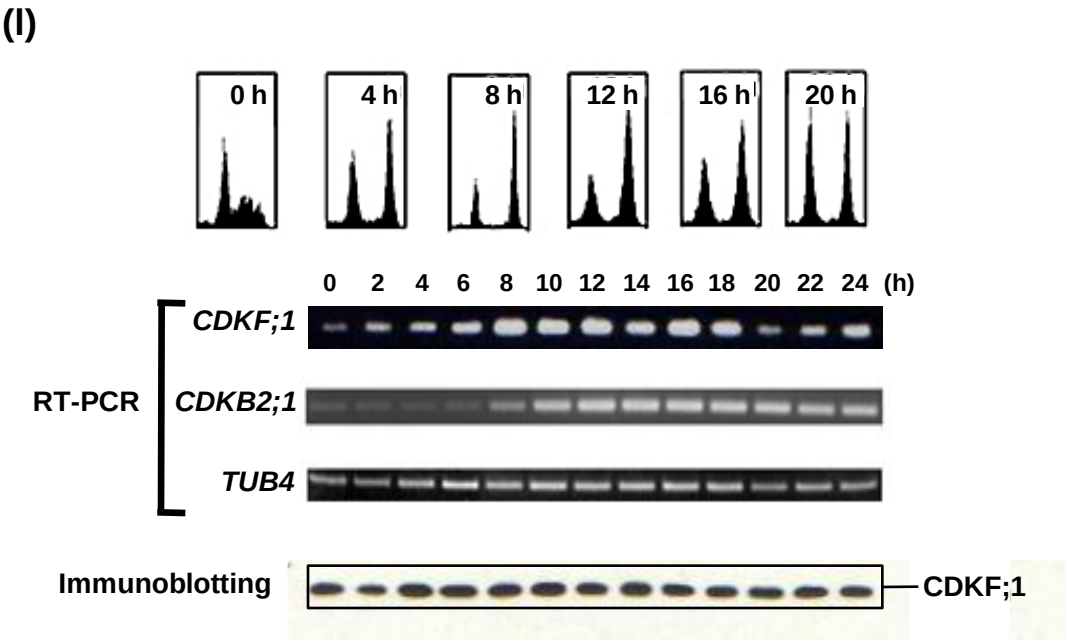

(l)
0 h
4 h
8 h
12 h
16 h
20 h
0 2 4 6 8 10 12 14 16 18 20 22 24 (h)
RT-PCR
CDKF;1
CDKB2;1
TUB4
Immunoblotting
CDKF;1

Figure 2

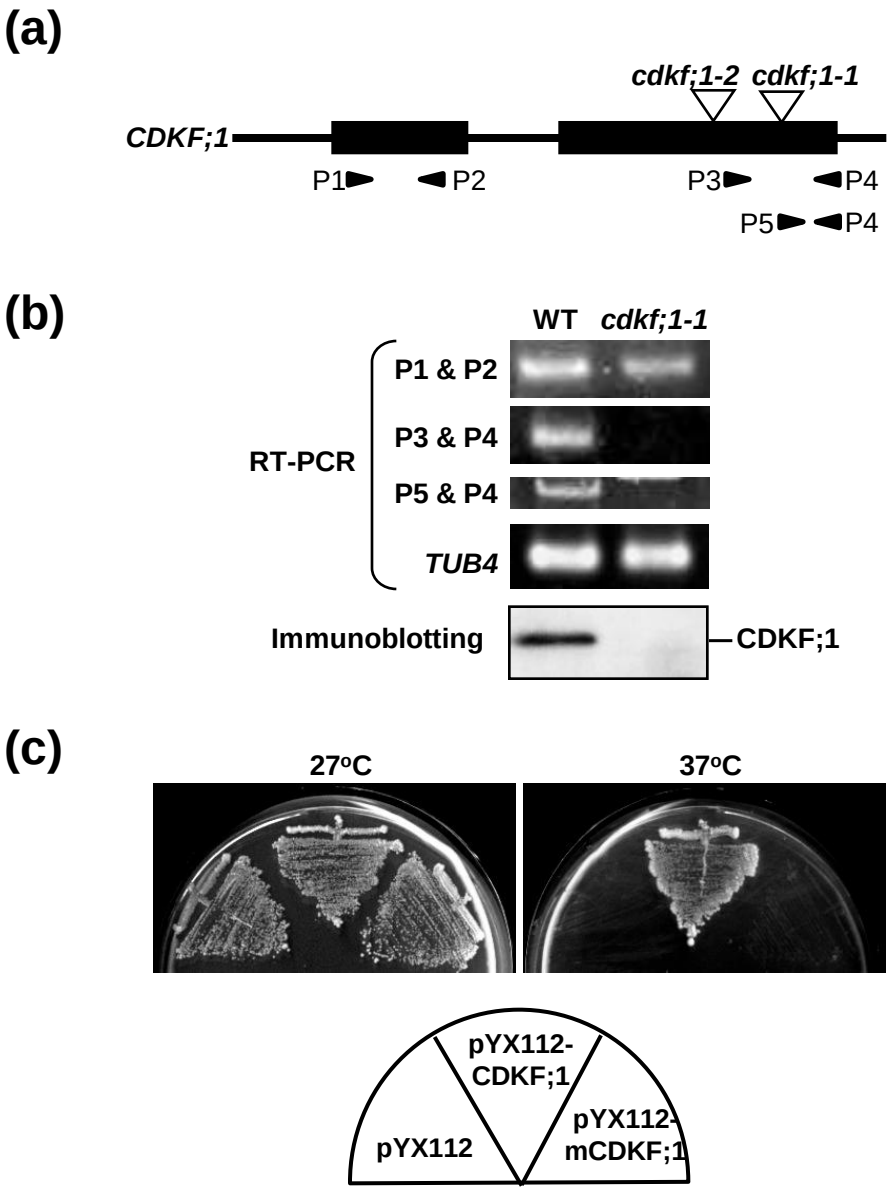

(a)
cdkf;1-2
cdkf;1-1
CDKF;1
P1
P2
P3
P4
P5
P4
(b)
WT
cdkf;1-1
P1 & P2
P3 & P4
P5 & P4
TUB4
RT-PCR
Immunoblotting
CDKF;1
(c)
27°C
37°C
pYX112-
CDKF;1
pYX112
pYX112-
mCDKF;1

Figure 3

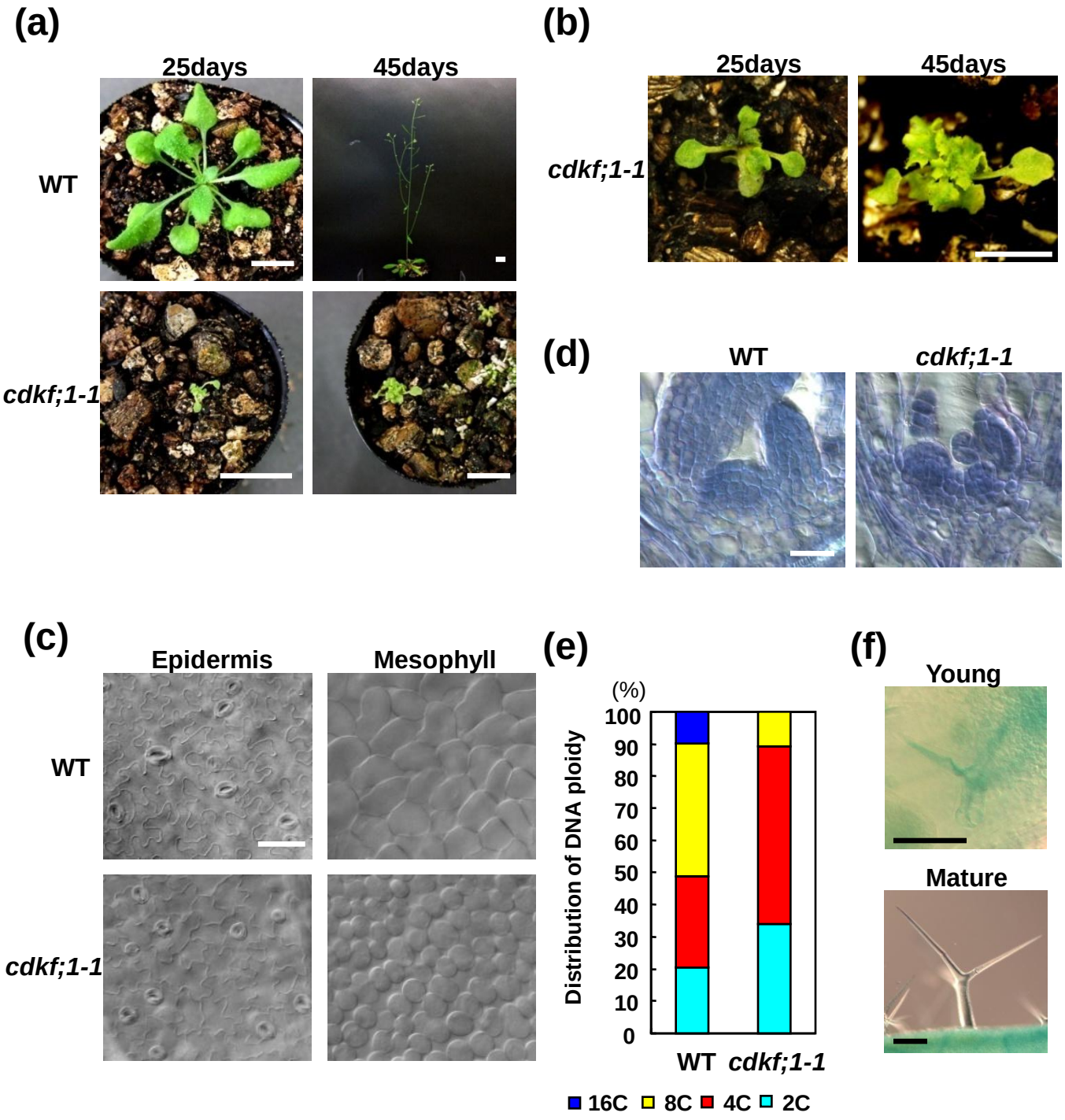

(a)
25days
45days
WT
cdkf;1-1
(b)
25days
45days
cdkf;1-1
(d)
WT
cdkf;1-1
(c)
Epidermis
Mesophyll
WT
cdkf;1-1
(e)
(%)
100
90
80
70
60
50
40
30
20
10
0
Distribution of DNA ploidy
WT
cdkf;1-1
16C
8C
4C
2C
(f)
Young
Mature

Figure 4

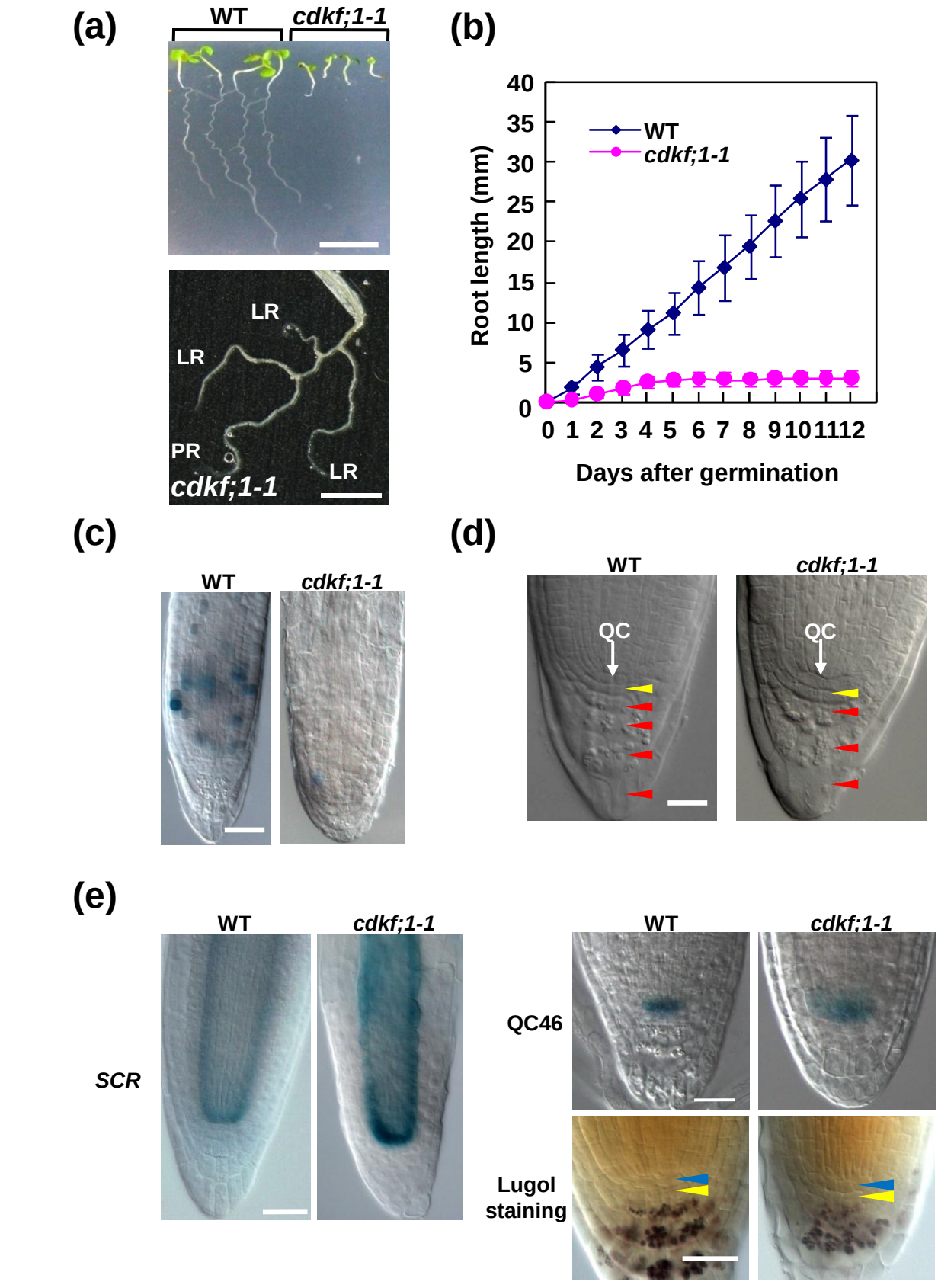

(a)
WT
cdkf;1-1
LR
LR
PR
LR
cdkf;1-1
(b)
Root length (mm)
Days after germination
WT
cdkf;1-1
(c)
WT
cdkf;1-1
(d)
WT
cdkf;1-1
QC
QC
(e)
WT
cdkf;1-1
SCR
WT
cdkf;1-1
QC46
Lugol staining

Figure 5

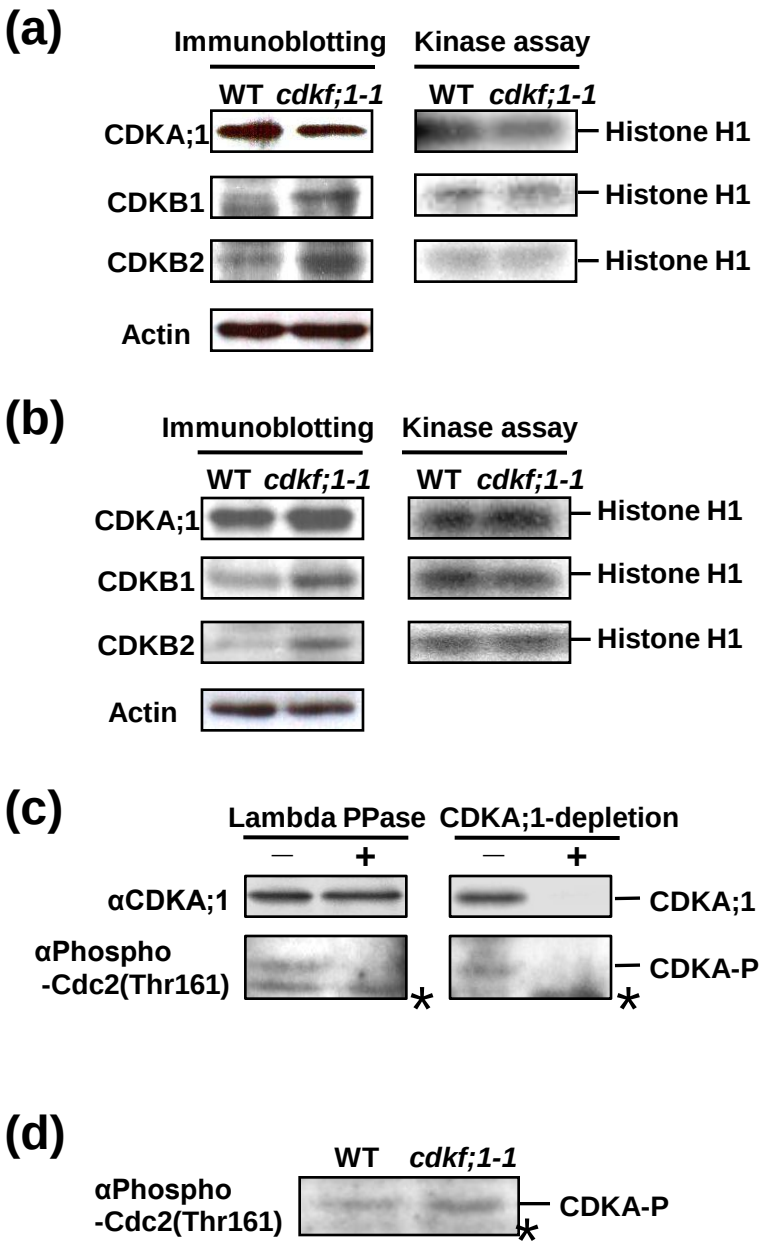

(a)
Immunoblotting
Kinase assay
WT
cdkf;1-1
WT
cdkf;1-1
CDKA;1
CDKB1
CDKB2
Actin
Histone H1
Histone H1
Histone H1
(b)
Immunoblotting
Kinase assay
WT
cdkf;1-1
WT
cdkf;1-1
CDKA;1
CDKB1
CDKB2
Actin
Histone H1
Histone H1
Histone H1
(c)
Lambda PPase
CDKA;1-depletion
–
+
–
+
αCDKA;1
αPhospho
-Cdc2(Thr161)
CDKA;1
CDKA-P
*
*
(d)
WT
cdkf;1-1
αPhospho
-Cdc2(Thr161)
CDKA-P
*

Figure 6

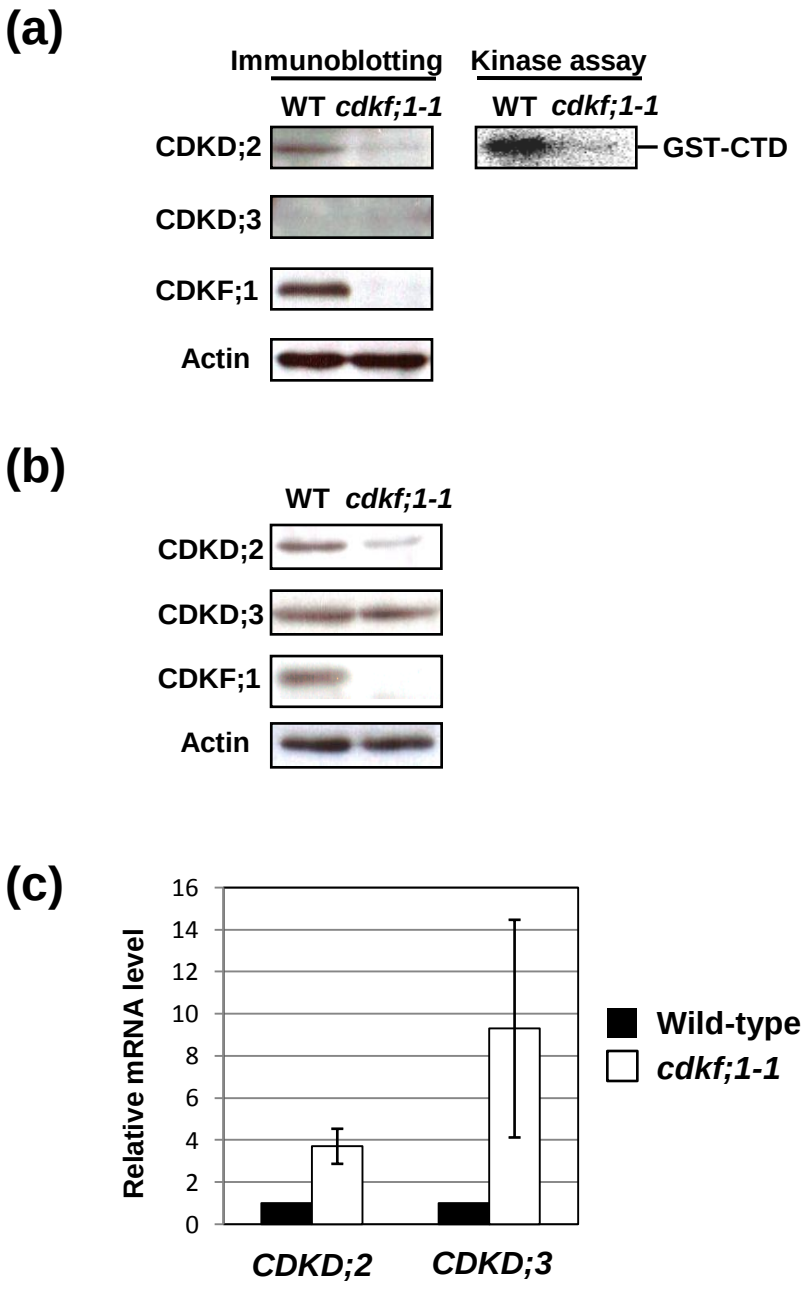

(a)
Immunoblotting
Kinase assay
WT
cdkf;1-1
WT
cdkf;1-1
CDKD;2
GST-CTD
CDKD;3
CDKF;1
Actin
(b)
WT
cdkf;1-1
CDKD;2
CDKD;3
CDKF;1
Actin
(c)
Relative mRNA level
16
14
12
10
8
6
4
2
0
CDKD;2
CDKD;3
Wild-type
cdkf;1-1

Figure 7

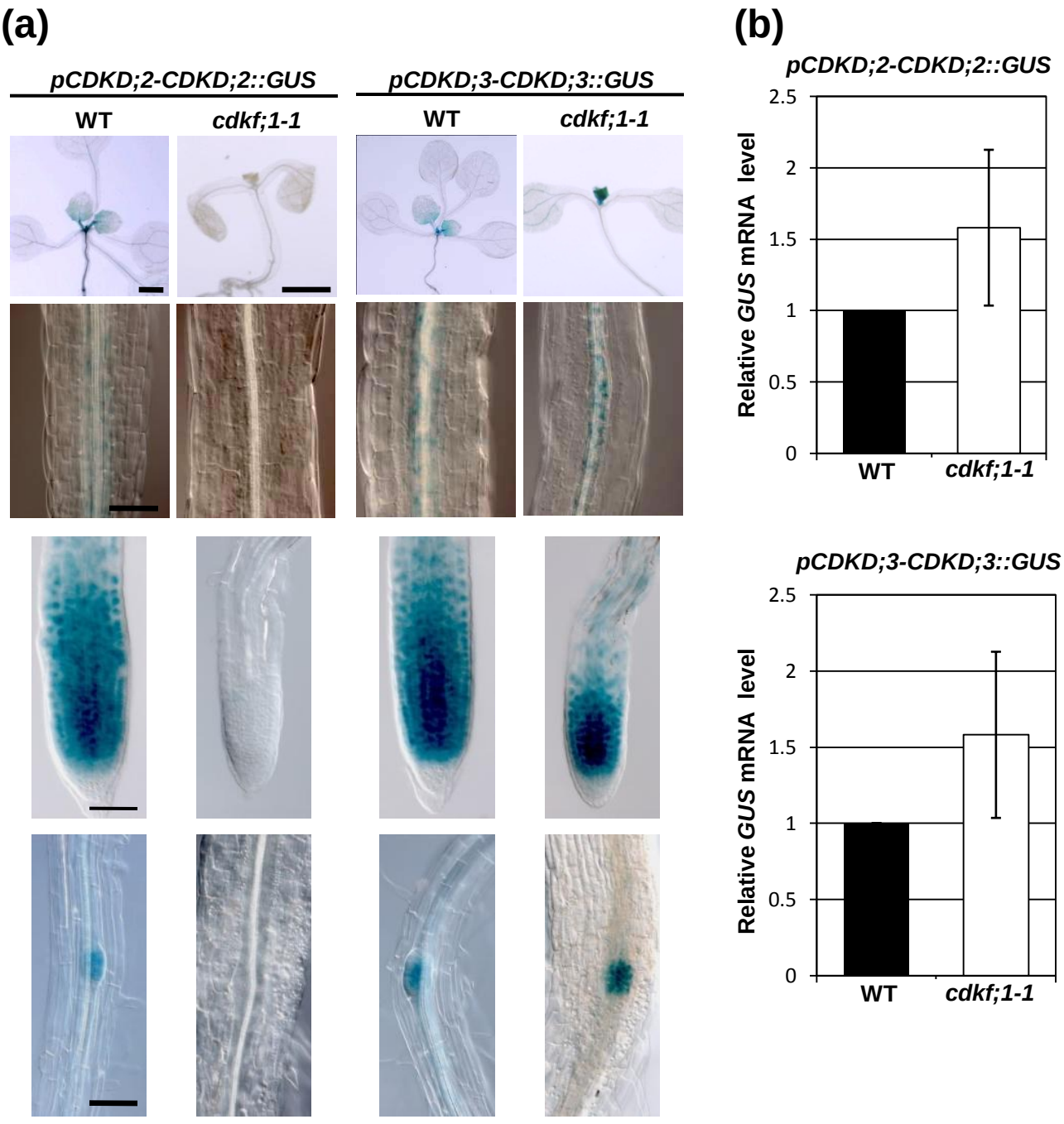
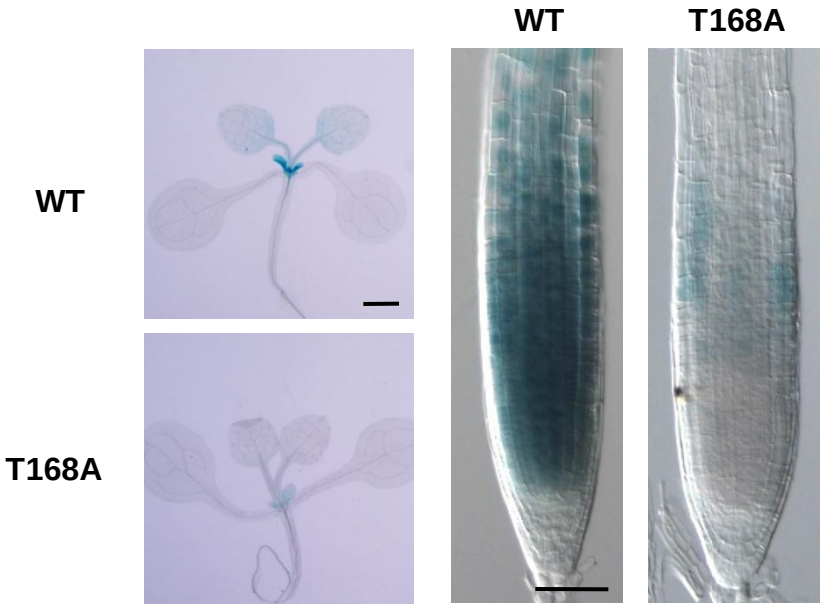
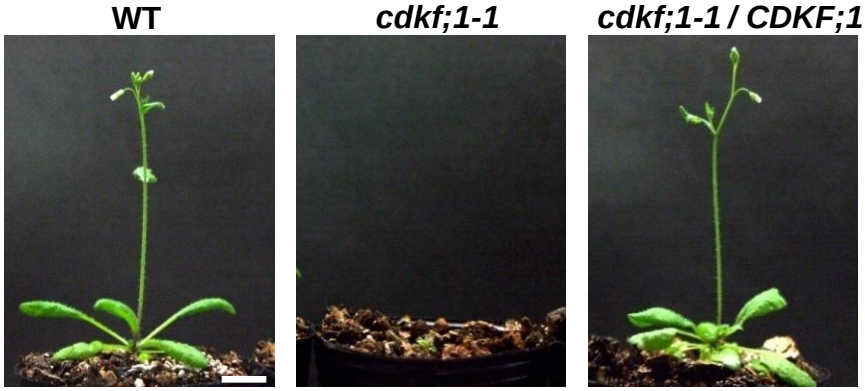

(a)
pCDKD;2-CDKD;2::GUS
pCDKD;3-CDKD;3::GUS
WT
cdkf;1-1
WT
cdkf;1-1
(b)
pCDKD;2-CDKD;2::GUS
Relative GUS mRNA level
2.5
2
1.5
1
0.5
0
WT
cdkf;1-1
pCDKD;3-CDKD;3::GUS
Relative GUS mRNA level
2.5
2
1.5
1
0.5
0
WT
cdkf;1-1

Figure 8



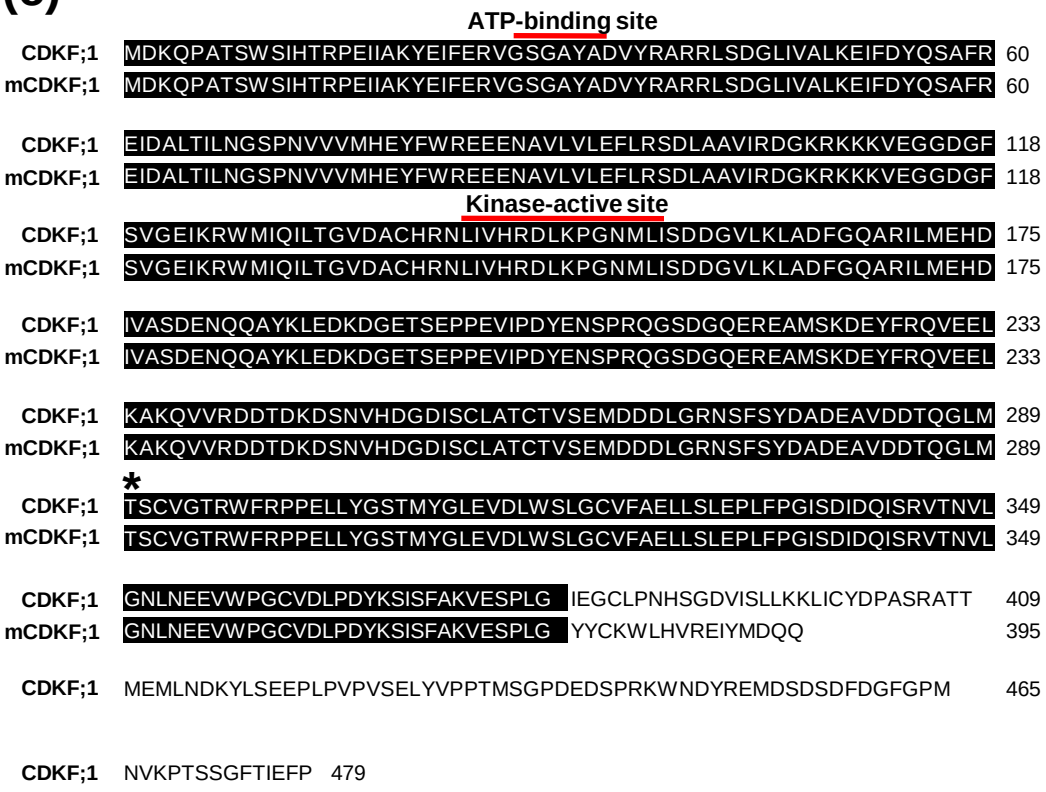
Supplemental Figure 1

(a)



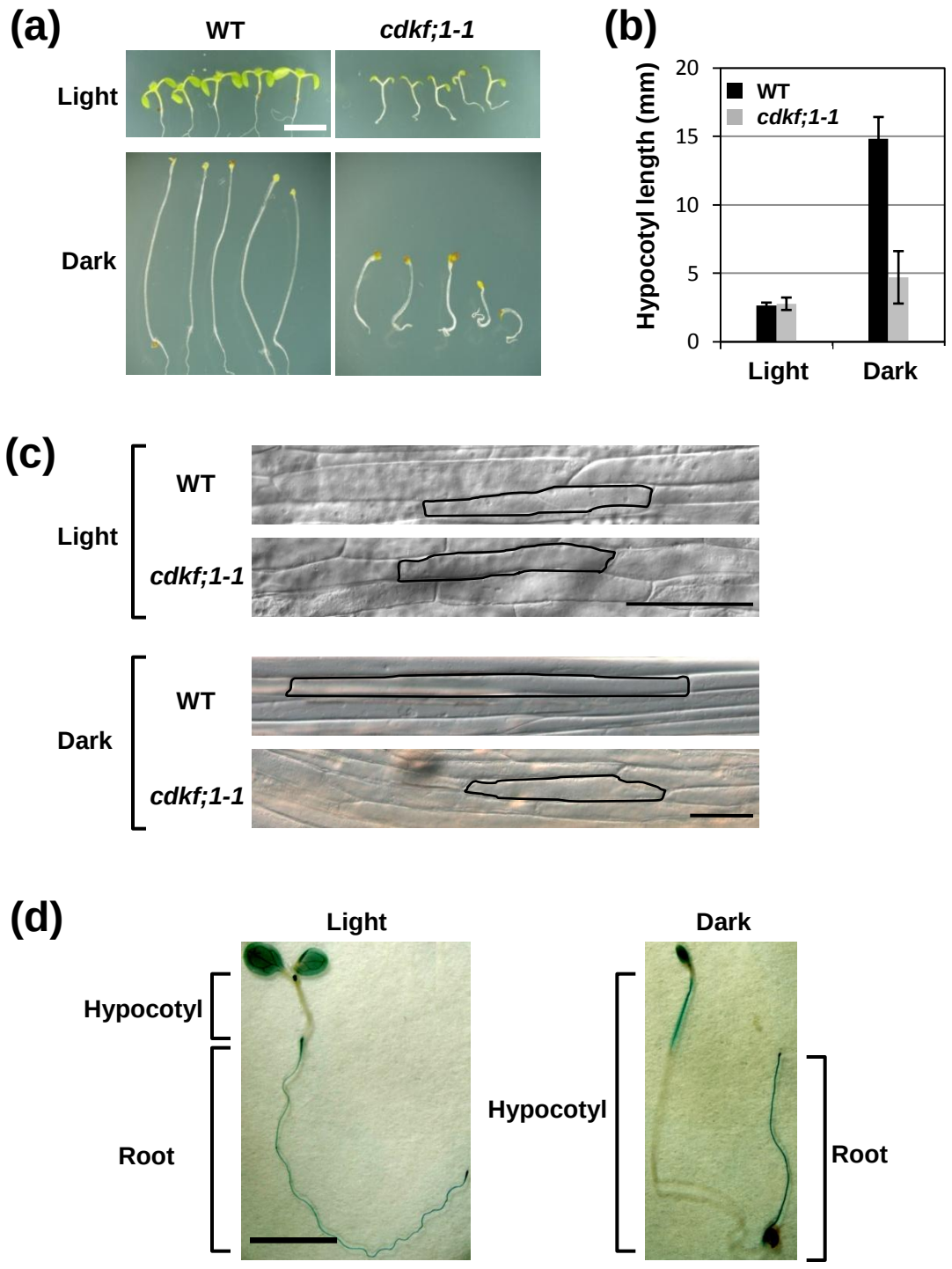
(b)

(c)



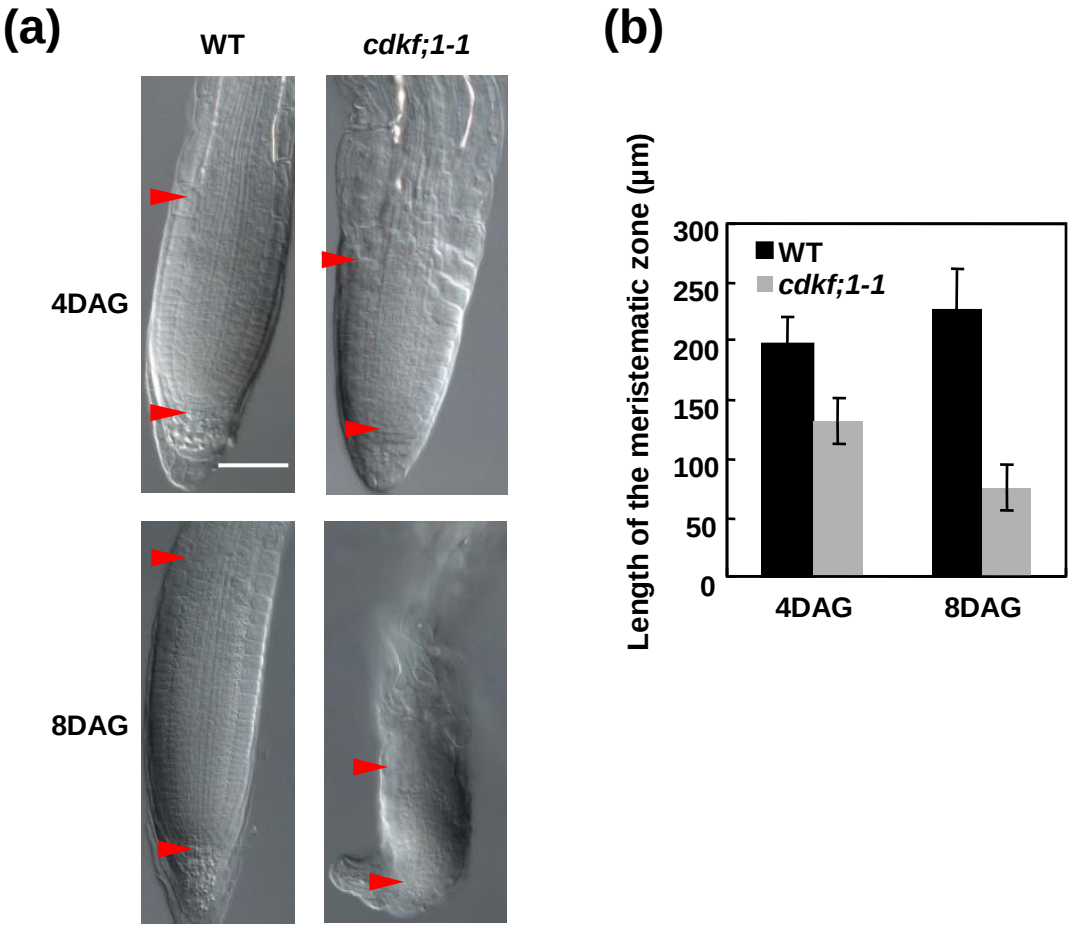
Supplemental Figure 1. Complementation of the *cdkf;1* mutants.
(a) Complementation of the *cdkf;1-1* mutants with the *CDKF;1* genomic fragment. *Arabidopsis* plants (32 DAG) are shown. Bar = 1 cm. (b) Junction sequences of the T-DNA insertion site. Five unknown nucleotides are inserted between the sequences of *CDKF;1* and the T-DNA left border. (c) Alignment of amino acid sequences of CDKF;1 and mCDKF;1. The asterisk indicates the conserved threonine residue within the T-loop.

Supplemental Figure 2



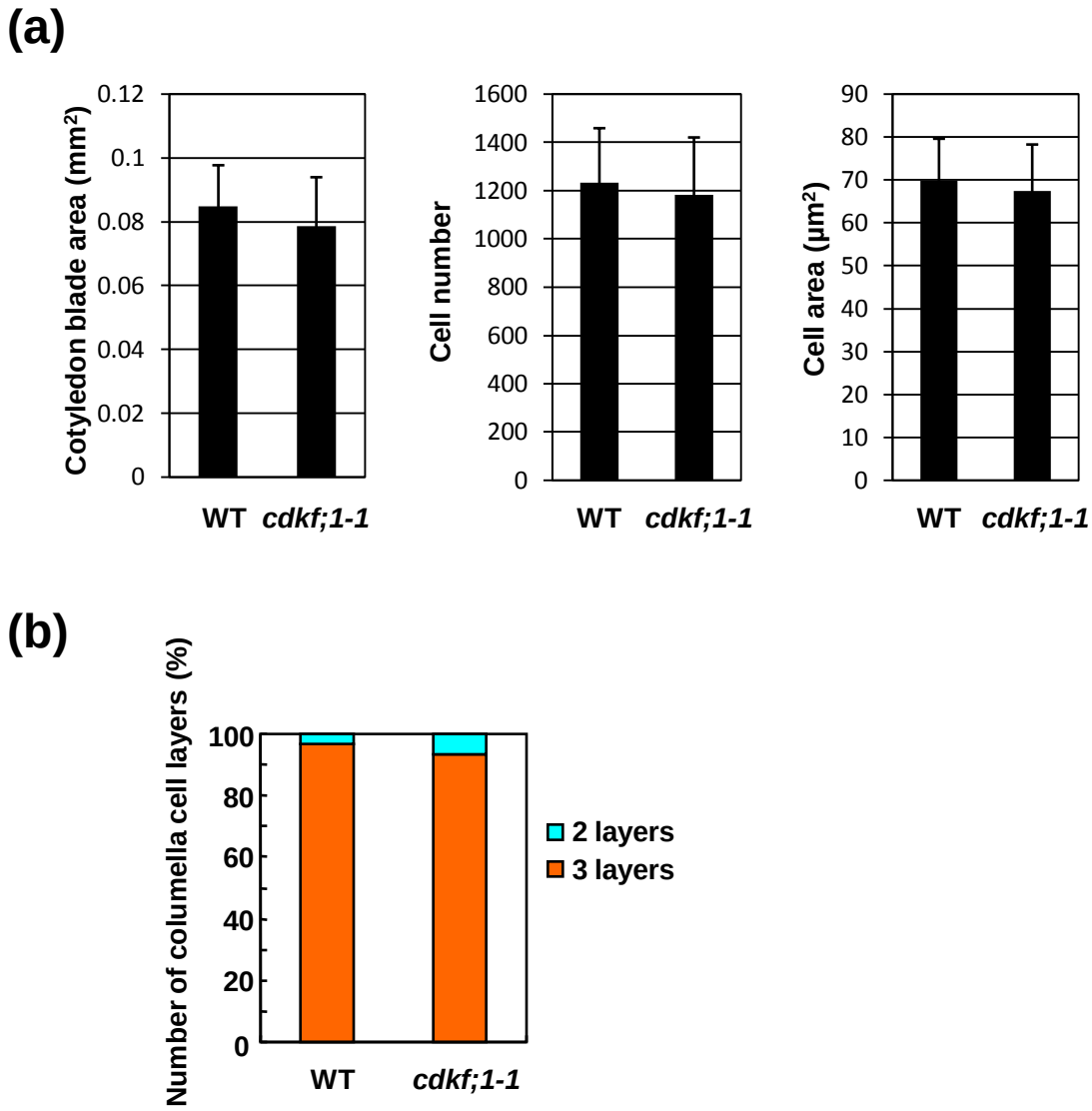
Supplemental Figure 2. Inhibition of hypocotyl elongation in the *cdkf;1-1* mutants. (a) 8-day-old seedlings of wild-type plants and the *cdkf;1-1* mutants grown under light or dark conditions. Bar = 5 mm. (b) Hypocotyl length of wild-type plants (black bars) and the *cdkf;1-1* mutants (grey bars) shown in (a). (c) Hypocotyl epidermal cells of wild-type plants and the *cdkf;1-1* mutants shown in (a). Bar = 100 μm. (d) GUS staining of 8-day-old seedlings harbouring *pCDKF;1-CDKF;1::GUS*. Bar = 5 mm.

Supplemental Figure 3



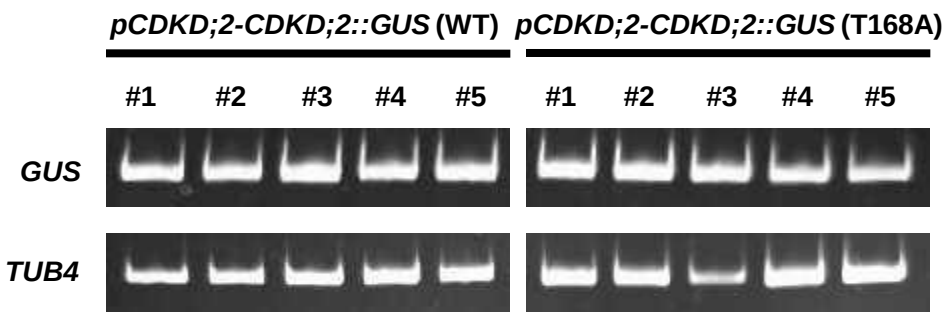
Supplemental Figure 3. Root meristem size is reduced in the *cdkf;1-1* mutants.
(a) Red arrowheads indicate the meristematic zone in primary roots. Bar = 100 μm. (b) The distance from the quiescent centre to first vacuolated cells was measured. Data are presented as mean ± SE of 17 samples.

Supplemental Figure 4



Supplemental Figure 4. Embryogenesis is not defective in the *cdkf;1-1* mutants.
(a) Blade area, cell number and cell area of cotyledons were measured with mature embryos of dry seeds obtained from wild-type plants and the heterozygous *cdkf;1-1* mutants. Data are presented as mean ± SE of 25 samples for wild-type plants and 89 samples for the *cdkf;1-1* mutants. (b) Number of columella cell layers in mature embryos was counted. Data are presented as mean ± SE of 160 samples.

Supplemental Figure 5



Supplemental Figure 5. RT-PCR analysis of *pCDKD;2-CDKD;2::GUS* transgenic plants.
RT-PCR was conducted with total RNA from 10-day-old seedlings of transgenic plants harbouring *pCDKD;2-CDKD;2::GUS* (WT) or *pCDKD;2-CDKD;2::GUS* (T168A) by using primers for *GUS* and *TUB4*. Five independent lines were examined for each transgenic plant.

Supplemental Table 1 Primers used for RT-PCR

Name	Sequence (5' > 3')
CDKB2;1 forward	ATGGACGAGGGAGTTATAGCAGTTTC
CDKB2;1 reverse	TCAAAAACCAGGTACAGTACAGTTTTGCC
CDKD;2 forward	GATATTAAGTCTTATATGTTG
CDKD;2 reverse	CTCAGGCGCTCTGTACCATGTAG
CDKD;3 forward	AAGCCAAATAACTTGTTAAT
CDKD;3 reverse	AAGCTTTGTTAAATCCGGCC
CDKF;1 forward	AAGAAGGTAGAAGGAGGGGATGGAT
CDKF;1 reverse	CATTTCACTAACAGTGCACGTTGCA
TUB4 forward	AGAGGTTGACGAGCAGATGA
TUB4 reverse	CCTCTTCTTCCTCCTCGTAC
CDKD;2 forward (Real-time)	AAGCCACCGACACAAAGACT
CDKD;2 reverse (Real-time)	GTAGCTTGATTTCTCTTAAAGCTGTG
CDKD;3 forward (Real-time)	AAGCAGGCTCTAGAACACAGGTA
CDKD;3 reverse (Real-time)	GGCTTTGGGAGCTTAGCC
ACT8 forward (Real-time)	CTAAACTAAAGAGACATCGTTTCCA
ACT8 forward (Real-time)	GTTTTTATCCGAGTTTGAAGAGGCT
GUS forward (Real-time)	TTAACTATGCCGGAATCCATCGC
GUS reverse (Real-time)	AACGCTGACATCACCATTGGC
GUS forward (RT-PCR)	ACTGAACTGGCAGACTATCC
GUS forward (RT-PCR)	ACGATGCCATGTTCATCTGC
P1	ACGAATACTTCTGGCGTG
P2	ACTGCTTCGTAGCATCG
P3	GTTAGTGAAATGGATGATGATCTCGG
P4	CACTACTGGTAGGCTTTACATTCATGGGT
P5	GAGGATTCTCCGAGAAAGTGGAATG